Sample dilution and bacterial community composition influence empirical leucine-to-carbon conversion factors in surface waters of the world's oceans

by

Eva Teira (1), Víctor Hernando-Morales (1), Francisco M Cornejo-Castillo (2), Laura Alonso-Sáez (3,8), Hugo Sarmento (4), Joaquín Valencia-Vila (5), Teresa S Catalá (6), Marta Hernández-Ruiz (1), Marta M Varela (5), Isabel Ferrera (2), Xosé Anxelu G Morán (7, 8), Josep M Gasol (2)

(1) Universidade de Vigo, Vigo, Spain

(2) Institut de Ciències del Mar, CSIC; Barcelona, Spain

(3) AZTI, Sukarrieta, Spain

(4) Department of Hydrobiology, Federal University of São Carlos, São Carlos, SP - Brazil

(5) Instituto Español de Oceanografía, Centro Oceanográfico de A Coruña, A Coruña, Spain

(6) Universidad de Granada, Granada, Spain

(7) Red Sea Research Center, Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia

(8) Instituto Español de Oceanografía, Centro Oceanográfico de Xixón, Xixón, Spain

Abstract

The transformation of leucine incorporation into prokaryotic carbon production rates requires the use of either theoretical or empirically determined conversion factors. Empirical leucine-to-carbon conversion factors (eCFs) vary widely across environments, and little is known about their potential controlling factors. We conducted 10 surface seawater manipulation experiments across the world's oceans, where the growth of the natural prokaryotic assemblages was promoted by filtration (i.e. removal of grazers; F treatment) or filtration combined with dilution (i.e. relieving also resource competition; FD treatment). The impact of sunlight exposure was also evaluated in the FD treatments, and we did not find a significant effect on the eCFs. The eCFs varied from 0.09 to 1.47 kg C mol Leu^{-1} and were significantly lower in the filtered and diluted (FD) than in the filtered (F) treatments. Also, changes in bacterial community composition during the incubations, as assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA), were stronger in the FD than in the F treatments, as compared to unmanipulated controls. Thus, we discourage the common procedure of diluting samples (in addition to filtration) for eCFs determination. The eCFs in the filtered treatment were negatively correlated with the initial chlorophyll *a* concentration, picocyanobacterial abundance (mostly *Prochlorococcus*) and the percentage of heterotrophic prokaryotes with high nucleic acid content (%HNA). The latter two variables explained 80% of the eCFs variability in the F treatment, supporting the view that both *Prochlorococcus* and HNA prokaryotes incorporate leucine in substantial amounts although resulting into relatively low carbon production rates in the oligotrophic ocean.

INTRODUCTION

Prokaryotic heterotrophic production, (PHP) also known as bacterial production, is a key variable for evaluating the role of prokaryotes in ocean carbon fluxes. However, direct PHP measurements by means of biomass increase with time require long incubations (several days). This procedure is excessively time-consuming for routine measurements at adequate spatial and temporal scales and, therefore, PHP is typically estimated from related metabolic processes. The incorporation rates of radiolabelled substrates such as leucine or thymidine are by far the most widespread approaches due to their high sensitivity and the short incubation times required (1-2). However, the transformation of leucine or thymidine incorporation rates into rates of prokaryotic carbon production relies on the use of conversion factors (CFs). In the case of leucine, a theoretical CF of 3.1 kg C mol Leu^{-1} was estimated by Simon and Azam (3) based on the protein content of an average bacterial cell and the typical ratio of carbon-to-protein content, assuming a 2-fold dilution with external leucine (or 1.55 kg C mol Leu^{-1} assuming no isotope dilution). Regardless of the systematic application of any of these two theoretical CF in most published studies, compelling evidence indicates that the relation between leucine incorporation and carbon produced is far from constant, and thus, the variability in empirically determined CFs is large (4-8).

The determination of empirical CFs (eCFs) typically involves the facilitation of bacterial net growth by incubation of natural prokaryotic assemblages for up to several days (until entering stationary phase) in the dark and after reducing grazing pressure and/or increasing resource availability by dilution and/or filtration, with or without added nutrients (2, 9, 10). It is not clear to what extent the experimental design may influence the obtained eCFs. For example, several studies found lower eCFs in glucose and/or inorganic nutrient amended compared to unamended incubations (9-11), which

78 suggests that a strong dilution, substantially increasing resource availability, may
79 produce lower estimates of the leucine-to-carbon CF. The incubations under dark
80 conditions may also affect the derived eCF as sunlight has been shown to have a
81 relevant impact on bacterial metabolism (12-14). However, to the best of our
82 knowledge, the effect of light on the determination of eCFs has never been assessed.

83 On top of the variability of eCFs associated to methodological aspects, several
84 studies have shown variation in relation to ecological factors, such as resource
85 availability (5, 15), chlorophyll-*a* concentration (11), prokaryotic growth efficiency (4,
86 6, 16), or bacterial community composition (11). Yet, only a few of these studies were
87 conducted in open ocean surface waters (4, 6), which might hamper our ability to derive
88 an empirical model able to predict CFs from environmental variables in vast extensions
89 of the global ocean. Indeed, the wider the gradient of environmental conditions
90 surveyed, the wider the range of CFs found. As an example, CFs varied two orders of
91 magnitude along a trophic gradient from shelf-break upwelling to oligotrophic open
92 ocean Atlantic waters (4), and leucine-to-carbon CFs were only significantly correlated
93 with bacterial growth efficiency, a non-routinely measured variable. While the few
94 published leucine-to-carbon CFs in surface oligotrophic oceanic waters are consistently
95 low (4, 6, 17), no studies on the large-scale variability of CFs in relation to
96 environmental factors have been conducted so far in these low productive areas.

97 The aim of our work was to evaluate the effect of filtration, dilution and sunlight
98 exposure on leucine-to-carbon CF estimates, and to relate the empirically derived CFs
99 with environmental factors in surface oceanic oligotrophic waters of the world's oceans,
100 during the Malaspina 2010 circumnavigation expedition, designed to cover a whole
101 longitudinal range of tropical and subtropical waters between 30ºN and 30ºS.

MATERIALS AND METHODS

Sample collection and experimental setup. Experiments were carried out at 10 stations located in the tropical and subtropical Atlantic, Indian and Pacific oceans between 14th December 2010 and 14th July 2011 during the Malaspina 2010 circumnavigation expedition on board the R/V Hespérides (Fig. 1). Conductivity–Temperature–Depth (CTD) casts were carried out at each station with a Sea-Bird Electronics 911 plus probe attached to a rosette equipped with Niskin bottles. Samples for phosphate and nitrate measurements were frozen and their concentrations were determined by standard colorimetric methods with a Technicon autoanalyzer. Chlorophyll-*a* concentration was fluorometrically determined after biomass concentration onto 0.2 μm pore size polycarbonate filters and extraction in 90% acetone.

Seawater for the experiments was collected at 3 m depth using 30 L Niskin bottles. Ultraviolet radiation transparent 3 L carboys were used for the incubations. Each experiment consisted of 3 treatments (in duplicate): a filtered treatment through 0.8 μm pore size polycarbonate filters to remove large predators while maintaining most free-living prokaryotes and exposed to sunlight (LF standing for light + filtration)+), a filtered and diluted (0.8 μm filtered seawater diluted (1:5) with 0.2 μm filtered seawater) treatment to reduce both predators and resource competition and exposed to sunlight (LFD standing for light + filtration + dilution) and a filtered and diluted treatment kept under dark conditions (DFD standing for dark + filtration + dilution). A control, consisting on unmanipulated seawater exposed to sunlight (LC standing for light control) was also incubated in order to check for changes in bacterial community composition associated with sample manipulation. LC, LF and LFD samples were

126 incubated on deck under natural light conditions, and DFD was incubated on deck in
127 dark conditions. The experimental carboys were kept at near *in situ* temperature by
128 circulating surface seawater in the incubation tank. The experiments lasted 3 days and
129 samples were taken every 12-24 h for heterotrophic prokaryotic biomass (HPB) (as
130 estimated by flow cytometry) and leucine incorporation rate measurements. The
131 integrative method was used for the leucine-to-carbon conversion factor calculation (18)
132 using the time intervals where an increase in heterotrophic prokaryotic biomass was
133 observed. The conversion factor was calculated as the HPB (Kg C L^{-1}) produced over
134 the selected time period of the experiment (essentially, the final HPB minus the initial
135 HPB for that time period) divided by the total amount of leucine (mol Leucine L^{-1})
136 incorporated during that selected time period. The total amount was measured by
137 integrating the incorporation rates over the selected time period.

138 Bacterial community composition was assessed at the beginning and at the end
139 of the experiments using the ARISA (Automated Ribosomal Intergenic Spacer Analysis)
140 fingerprinting technique (see below).

141 **Flow cytometry analyses.** Samples were fixed and processed with a FACSCalibur
142 flow cytometer (BD-Biosciences) with a blue laser emitting at 488 nm. Samples of 1.2
143 mL of seawater were fixed with a paraformaldehyde-glutaraldehyde mix (1% and
144 0.05% final concentrations, respectively) and stored at -80ºC until analysis in the
145 laboratory within a maximum of seven months after the end of the cruise. Samples were
146 stained with SYBRGreen I, at a final concentration of 1:10.000, for 15 min in the dark
147 at room temperature. The average flow rate used was 12 μL min^{-1} and acquisition time
148 ranged from 30 to 260 seconds depending on cell concentration in each sample. Data
149 were inspected in a FL1 versus light side scatter (SSC, also termed right-angle light
150 scatter (RALS)) plot and analyzed as detailed in Gasol and del Giorgio (19), including

151 the differentiation of the two widespread groups of low nucleic acid containing (LNA)
152 and high nucleic acid containing (HNA) prokaryotes. Molecular Probes latex beads (1
153 μm) were always used as internal standards. The biovolume of prokaryotic cells was
154 estimated using the calibration obtained by Calvo-Díaz and Morán (20) relating relative
155 light side scatter (population SSC divided by bead SSC) to cell diameter assuming
156 spherical shape. Cell biovolume was finally converted into carbon biomass with the
157 equation of Gundersen et al. (21): cell biomass (fg C $cell^{-1}$) = 108.8 * $V^{0.898}$.
158 *Prochlorococcus* picocyanobacteria, which tended to overlap partially with the HNA
159 prokaryote cluster, were subtracted from total heterotrophic prokaryotic counts by
160 independent assessment in non-stained aliquots. *Synechococcus* and *Prochlorococcus*
161 cyanobacteria were in turn identified in plots of SSC *versus* red fluorescence (FL3,
162 >650 nm), and orange fluorescence (FL2, 585 nm) versus FL3. Picocyanobacterial
163 biomass was calculated by using the following volume-to-carbon conversion factors:
164 230 fg C μm^{-3} for *Synechococcus* and 240 fg C μm^{-3} for *Prochlorococcus* (22).

165 **Leucine incorporation rates.** The [^{3}H]leucine incorporation method (23),
166 modified as described by Smith and Azam (24), was used to determine leucine
167 incorporation rates. From each experimental carboy, six 1.5 mL vials (4 replicates and 2
168 killed controls) were filled with 1.2 mL of seawater. A total of 120 μL of cold 50%
169 trichloroacetic acid (TCA) were added to the killed controls. After 15 minutes, 20 nmol
170 L^{-1} of L-[4,5-^{3}H] leucine (144.2 Ci $mmol^{-1}$, Amersham) was added to all samples which
171 were incubated for 2.5-6 h in the same incubation tank and under the same light
172 conditions as the corresponding experimental carboys.

173 **Automated ribosomal intergenic spacer analysis (ARISA).** Automated
174 ribosomal intergenic spacer analysis (ARISA) was conducted with DNA extracted from
175 samples taken at the beginning and the end of each experiment. 1-2 L seawater samples

were pre-filtered through a 3 μm pore-size filter and subsequently filtered through a 0.2 μm pore-size polycarbonate filters (Nuclepore Whatmann, 47-mm filter diameter). Filters were then stored at -80ºC until DNA extraction. Microbial community DNA was extracted using Ultra Clean Soil DNA isolation kit (MoBio Laboratories, Inc.) and quantified in a Nanodrop. Bacterial ARISA was performed using the ITSF/ITSReub primer set (Thermo Scientific) previously described by Cardinale et al. (25). The PCR reaction (25 μL) contained final concentrations of 1x PCR buffer (Genecraft), 2.5 mmol L^{-1} $MgCl_2$ (Genecraft), 250 μmol L^{-1} of each dNTP (Genecraft), 250 nmol L^{-1} of universal primer ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and eubacterial ITSReub (5'-GCCAAGGCATCCACC-3') (8), the former being labelled at the 5' end with the fluorescein amidite dye (6-FAM), 40 ng $\mu\Lambda^{-1}$ bovine serum albumin, 3.5 U of BioThermD-™ Taq DNA Polymerase (GeneCraft) and approx. 0.13 ng μL^{-1} of template DNA. The reaction mixture was held at 94ºC for 2 min followed by 32 cycles of amplification at 94ºC for 15 s, 55ºC for 30 s and 72ºC for 3 min, with a final extension of 72ºC for 10 min. The PCR reactions were conducted in duplicate for each DNA extract (this compensates for any anomalously running fragments both in the samples as well as in the standards). Amplification products were sent for capillary electrophoresis migration on a 50-cm-capillary ABI Prism 3730XL DNA analyzer (Applied Biosystems) at Genoscreen (www.genoscreen.fr/). A standardized migration cocktail contained 0.5 μl of amplification product, 0.25 μl of internal size standard LIZ 1200 (20-1200 pb, Applied Biosystems) and 8.75 μL of deionized Hi-Di formamide (Applied Biosystems). The mixture was denatured 5 min at 95°C and kept on ice before being further processed by the sequencer. Capillary electrophoresis parameters were as following: 10 kV (run voltage), 1.6 kV (injection voltage), 22 s (injection time) and 63°C (oven temperature). Resulting electropherograms were analyzed using the DAx

201 software (Data Acquisition and Analysis Software, Van Mierlo Software). Internal size
202 standards were built by using a second-order least-squares method and local Southern
203 method. Profiles were double checked manually for perfect internal size standard fit and
204 stable baselines. Baselines were then extracted, and subsequently, peak sizes heights
205 and absolute areas were determined. The same process was done for the PCR negative
206 sample. From the negative, the percentile 95 was calculated for the height measurement,
207 and used as a threshold. Samples with peak heights below percentile 95 were discarded
208 (95 percentile of each duplicated PCR negative presented values of 9 and 8.7 relative
209 fluorescence intensity (RFI) respectively).

210 Profile peaks were binned and reordered by OTUs (operational taxonomic unit)
211 by using R automatic binning and interactive binning scripts (26). Binning was carried
212 out independently of the sample (peaks from all samples together). To avoid size calling
213 imprecisions, a window size (WS) of 2 bp (determined by preliminary empirical tests)
214 was used for the binned method, and only peaks on the range 200 to 1,200 bp and with
215 peak values above 0.09% of total RFI were taken into account. Peaks from duplicates
216 were manually checked using binned-OTU tables, to avoid erroneous OTU divisions
217 due to rearrangement of all samples together.

218 **Statistical analyses.** Repeated measures ANOVA (RMANOVA) test was
219 performed to evaluate the significance of the differences observed in the eCFs among
220 the different treatments (LF, LFD, DFD). The square root normalized OTU relative
221 abundances assessed with ARISA were used to calculate pairwise similarities in
222 bacterial community composition among samples based on the Bray-Curtis similarity
223 index. Similarity patterns among samples were examined using a hierarchical cluster
224 analysis. Dendrograms were generated using the group average method and the Simprof
225 test was used to test for differences between the generated clusters at the 95%

226 confidence level. All the ARISA statistical analyses were completed in PRIMER-E v6
227 (27).

228

229 **RESULTS**

230 **Initial conditions.** A considerably wide range of initial seawater conditions was
231 observed in the experiments, both for environmental variables (Table 1) and for
232 bacterial community composition (Fig. 2). Chlorophyll-*a* concentration in the unfiltered
233 seawater ranged from 0.03 μg L^{-1} in the South Atlantic (experiment 3) to 0.21 μg L^{-1} in
234 the western tropical Pacific (experiment 9) (Table 1). Phosphate concentration also
235 varied about 10-fold from 0.02 μM in the Indian Ocean to 0.32 μM in the equatorial
236 Pacific (Table 1). By contrast, nitrate concentration varied two orders of magnitude,
237 from 0.03 μM in the North Pacific to 2.28 μM in the equatorial Pacific (Table 1).
238 Leucine incorporation rates in the 0.8-μm filtered seawater varied by three orders of
239 magnitude, whereas prokaryotic heterotrophic biomass (PHB) and picocyanobacterial
240 abundance (PCA) varied by one order of magnitude (Table 1). Approximately 50% of
241 the picocyanobacteria in the original sample were present in the <0.8 μm fraction (data
242 not shown). *Prochlorococcus* represented >90% of the picocyanobacteria in all the
243 experiments except in experiment 9 (eastern tropical North Pacific), which was
244 dominated by *Synechococcus* (details not shown). Heterotrophic prokaryotes were
245 generally dominated by LNA cells, with % HNA cells ranging from 27 to 53%. The
246 initial bacterial community composition as assessed by ARISA showed significant
247 differences among sampling sites (Fig. 2) although there were no significant differences
248 in the bacterial assemblages among experiments 1, 2, 8 and 10 (samples from Atlantic
249 and North Pacific oceans), between experiments 6 and 7 (samples from equatorial and

South Pacific ocean) and between experiments 4 and 5 (samples from the Indian ocean). The highest bacterial community composition similarity (68%) was observed between the experiments conducted in the Indian Ocean. Bacterial community composition in the eastern tropical North Pacific (experiment 9) differed most from the rest of locations (< 35% similarity).

Empirical leucine-to-carbon conversion factors (eCFs). eCFs were determined by comparison of leucine incorporation rates with the increase in bacterial biomass during the experimental incubations (Fig. 3). The resulting eCFs ranged from 0.09 ± 0.01 to 1.47 ± 0.08 kg C mol Leu^{-1}, showing values close to, or higher than 1 kg C mol Leu^{-1} in the filtered treatments of 4 out of 10 experiments. Overall, eCFs were higher in the filtered compared to the filtered and diluted treatments (Fig. 4). The eCFs in the light + filtration (LF), light + filtration + dilution(LFD) and dark + filtration + dilution (DFD) treatments followed similar variability patterns (Fig. 4), although significant differences were found among treatments (RMANOVA test, $p = 0.018$). Pairwise comparisons showed that eCFs were significantly higher in the LF than in both LFD and DFD treatments (Bonferroni test, $p < 0.042$). No significant differences between LFD and DFD treatments were found (Bonferroni test, $p > 0.05$), although eCFs were lower in the light than in the dark treatments in experiments 8 and 10.

In order to relate the observed variability in eCFs with environmental parameters we conducted a correlation analysis (Table 2). The conversion factors in the FD treatment (either under light or dark conditions) did not significantly correlate to any of the considered variables. By contrast, the eCFs in the LF treatment showed significant and strong negative correlations to chlorophyll-*a* concentration, picocyanobacterial abundance and the percentage of HNA prokaryotes (r ranging from -0.67 to 0.80, $p < 0.05$, Table 2). Significant semi-logarithmic or linear relationships were found between

275 the eCFs-LF and picocyanobacterial abundance (Fig. 5A) or the %HNA prokaryotes
276 (Fig. 5B), explaining 64% and 56% of the observed variability, respectively. A multiple
277 linear regression model including eCFs-LF as dependent variable and both %HNA
278 prokaryotes and log picocyanobacterial abundance as independent variables explained
279 80% of the variability in the eCFs-LF (eCFs = 4.98 [±1.04] – 0.73 [±0.25] × log
280 picocyanobacterial abundance – 0.021 [±0.09] × %HNA prokaryotes, $r^2 = 0.80$, adjusted
281 $r^2 = 0.75$, $p = 0.005$, $n = 10$) (standard errors in brackets). Log picocyanobacterial
282 abundance had a relatively greater effect on the eCFs-F (Beta coefficient = -0.57, p =
283 0.022) than %HNA prokaryotes (Beta coefficient= -0.46, p=0.049).

284 Bacterial community composition in the unmanipulated control treatment
285 showed, on average, 35% of similarity compared to the initial bacterial community
286 composition after 3 days of incubation (data not shown). The manipulation of nutrient
287 availability (by dilution), nutrient availability and grazing pressure (filtration and
288 dilution) and light caused changes in bacterial community composition (Fig.6).
289 However, the resulting community was mostly determined by the initial bacterial
290 assemblage, since the samples clustered primarily by experiment. Some experiments
291 showing similar eCFs also showed similar bacterial communities, such as experiments 1
292 and 2 or experiments 8 and 10 (Fig. 4 and 6). The effect of solar radiation in the
293 bacterial community composition was negligible in most of the diluted treatments, as no
294 significant differences were observed between LFD and DFD samples (Simprof test, $p <$
295 0.05), except in experiments 4, 8 and 10. The average similarity between LFD and DFD
296 samples was 63%. On the other hand, when comparing the unmanipulated control
297 samples (LC) with LF and LFD samples we observed that the effect of filtration alone
298 (LF) had fewer effects on bacterial community composition (average similarity of 60%)
299 than the combination of filtration and dilution (LFD) (average similarity of 46%).

300 Indeed, bacterial community composition in LF samples was not significantly different
301 to that in LC samples in 4 out of 8 experiments (Simprof test, $p < 0.05$) (Fig. 6). By
302 contrast, bacterial community composition in LFD samples did not significantly differ
303 from that in LC samples only in 1 out of 8 experiments (Simprof test, $p<0.05$) (Fig. 6).

304

305 **DISCUSSION**

306 Considering the widely demonstrated variability in eCFs, the use of constant
307 theoretical CFs may produce erroneous estimates of prokaryotic heterotrophic
308 production (PHP) by relying only on estimates of substrate incorporation rates rather
309 than also on the fate of the incorporated compounds (i.e. fraction of substrate that is not
310 assimilated into biomass) (6, 28). Several studies have shown that the use of theoretical
311 leucine-to-carbon CFs may overestimate both temporal and spatial variability in
312 prokaryotic biomass production (4-5). However, as CF experiments are time-consuming
313 and labor-intensive, unraveling which environmental factors drive changes in eCFs, and
314 deriving empirical models for predicting eCFs from basic environmental variables
315 represent a present challenge in aquatic microbial ecology. The broad range of
316 environmental conditions sampled in our survey (Table 2) allowed us to find an
317 empirical model that could be useful for deriving eCFs in surface oligotrophic oceanic
318 waters from flow cytometry data, the usual method for estimating microbial plankton
319 abundance.

320 **Filtration vs filtration and dilution in eCFs experiments.** Despite there are
321 several extensive studies reporting leucine-to-carbon eCFs across a variety of
322 environmental conditions, a meta-analysis is not easy to conduct due to the great
323 variability in the prokaryotic community pre-treatments. Early work by Coveney and

Wetzel (9) evaluating the effect of different pre-treatments (filtration, dilution and nutrient addition) on thymidine eCFs consistently found lower eCFs associated to the addition of phosphorus. In the case of leucine, only the study by Alonso-Sáez et al. (11) has systematically tested the effect of nutrient addition on the eCFs in prefiltered and 20x diluted samples from a coastal station along an annual cycle in the Mediterranean Sea. Significantly lower eCFs were found in inorganic nutrient enriched than in unamended seawater samples, and substantial changes in bacterial community composition were observed in association with nutrient additions. These authors hypothesized that addition of inorganic nutrients could lead to a situation of C limitation, where leucine would be utilized to obtain energy, leading to low net biomass production regardless of high leucine incorporation rates. Interestingly, the addition of carbon compounds such as glucose (10) or glucose and acetate (29) did not seem to affect leucine eCFs in studies performed in temperate coastal and Antarctic waters, respectively. Kirchman (30) also found that thymidine and leucine eCFs were not affected by additions of organic compounds in the subarctic Pacific.

Although we did not directly test the effect of nutrient enrichment, we did evaluate the effect of dilution of the sample with 0.2 μm prefiltered seawater, which can also increase nutrient availability. Most of the published leucine-to-carbon eCFs derive from filtered and diluted seawater incubations, with dilution factors ranging from 1x (10) to 20x (11); however the potential influence of dilution on the leucine-to-carbon eCFs estimations had not been tested so far. The significantly lower eCFs obtained in the filtered and diluted (LFD, DFD) compared to the filtered (LF) treatments is in agreement with the aforementioned negative effect of nutrient enrichment on eCFs estimates. Thus, our results suggest that dilution treatments may not be appropriate in CF experiments in oligotrophic areas. The effect of dilution was variable among the

experiments (Fig. 4), which may be related to the degree of nutrient limitation in each seawater incubation. According to the hypothesis of C limitation proposed by Alonso-Saéz et al. (11) and del Giorgio et al. (6), the effect of filtration and dilution on eCFs is expected to be higher when C is the primary limiting element. Under these conditions, the filtration and dilution increase mineral nutrient availability, which in turn may exacerbate C limitation as primary production is drastically reduced after filtration, leading to an uncoupling between leucine incorporation and bacterial biomass production (i.e low eCFs). The greatest difference between eCFs-LFD and eCFs-LF was observed in experiment 3, where the lowest chlorophyll-*a* concentration was measured (Table 1), suggesting that strong carbon limitation was the primary cause for the discrepancy.

A further argument that might discourage the dilution pretreatment in CF experiments in oligotrophic areas is the observed fact that the bacterial community composition developed in LFD treatments was significantly different than that in unmanipulated (LC) seawater, while LF samples were much more similar to the unmanipulated controls (Fig. 6). Despite both dilution and nutrient enrichment appear to affect leucine-to-carbon eCFs, we only found one study, conducted in Antarctic waters, where eCFs were estimated in filtered but non-diluted seawater incubations (29). The eCFs derived by these authors were very similar to those obtained by Teira et al. (31) in the same sampling region using the dilution approach. Summertime Southern Ocean waters are already nutrient-rich (29, 31) and increase of nutrient availability through dilution may have little effect on eCFs, supporting the role of the trophic status on the effect of the different experimental manipulations for estimating eCFs.

Only the variability in eCFs-LF could be explained by any of the environmental factors measured alongside in our study (Table 2). We are aware that other non-included

374 ecological factors might also have influenced the eCFs, such as DOC concentration,
375 leucine catabolism or bacterial growth efficiency (4, 6, 15). However, we believe that
376 the lack of a coherent explanation for the variability in eCFs in the diluted treatments
377 may be at least partially related to the inadequacy of the experimental design.
378 Considering the high variability that the methodological approach may introduce in
379 eCFs estimates, and in view of the large changes in bacterial communities and the lower
380 leucine yields associated with dilution, we strongly recommend avoiding dilution in
381 eCFs experiments, at least in oligotrophic waters.

382 **Relationship between eCFs and environmental factors.** Regardless of the
383 potential methodological problems, some potential explanations for the leucine-to-
384 carbon eCFs variability have been postulated to date. Overall, eCFs higher than the
385 theoretical (1.55 kg C mol leu^{-1}, assuming no isotopic dilution) can be explained by the
386 isotopic dilution of the radiotracer. If the radiotracer is not added at saturating
387 concentration, then the measured leucine incorporation rates will be lower than the
388 actual rates, artificially leading to high eCFs. This problem may typically occur in
389 coastal eutrophic waters where ambient leucine concentration may be higher than the
390 commonly used concentrations of added radiotracer (20-40 nmol L^{-1}). In fact, leucine-
391 to-carbon eCFs higher than the theoretical one have been repeatedly found at coastal
392 sites (6, 11, 32-34).

393 In open ocean oligotrophic waters, the leucine-to-carbon eCFs are consistently
394 lower than the theoretical one (4, 6, 17), which implies an unbalanced bacterial growth
395 in which net bacterial biomass production is low regardless of relatively high leucine
396 incorporation rates. We also measured eCFs lower than the theoretical one in all of the
397 experiments (Fig. 4). An unbalanced bacterial growth has been described under limiting
398 conditions, where protein synthesis is maintained in order to maximize survival rather

than growth and reproduction (15, 35-36), resulting in high turnover rates of intracellular protein (37). Yet, Alonso-Sáez et al. (4) measured relatively low protein turnover rates in oligotrophic waters of the subtropical Atlantic Ocean and concluded that the low eCFs found in the area were related to leucine catabolism by energy-limited bacterial cells. According to this hypothesis, leucine would be incorporated into the cell but a high portion would be respired before being used for protein synthesis; this would translate into low eCFs if the tritium signal of the incorporated and respired leucine were recovered by cold trichloroacetic acid. High percentages of leucine respiration (40-80%) associated to low eCFs have been found by Alonso-Sáez et al. (4) and del Giorgio et al. (6), rendering a significant negative correlation between the % of respired leucine and the eCFs ($r = 0.46$, $p < 0.001$, $n = 25$; pers. comm.).

Low leucine-to-carbon eCFs have been also related to a faster synthesis of transport proteins relative to cell duplication under low substrate availability by Calvo-Díaz et al (5). The later authors obtained a significant empirical model to predict eCFs from leucine incorporation rates and cellular carbon contents. However, samples were not prefiltered in their study, and thus grazing, although reduced by dilution, could have influenced their results. In our dataset, we did not find any significant correlation between eCFs and either leucine incorporation rates or bacterial cellular carbon content (Table 2). Moreover, by contrast with previous studies (11), we found a negative correlation between eCFs-LF and chlorophyll-a, indicating that higher eCFs occurred under limiting conditions. However, it is important to note that although we sampled a relatively wide range of environmental conditions, the trophic gradient was rather limited (chlorophyll a <0.21 mg m^{-3}) and thus energy limitation was supposedly occurring at all sampling sites.

423 The lower eCFs-LF associated to high picocyanobacterial abundance, dominated
424 by *Prochlorococcus*, could be related to the ability of these autotrophic bacteria to
425 incorporate leucine (38-41). As both picoautotrophs and heterotrophs contribute to
426 leucine assimilation, but only the biomass production of heterotrophs is taken into
427 account in the calculation of eCFs, a high abundance of picocyanobacteria during the
428 experiments may result in high bulk leucine incorporation rates irrespective of low
429 heterotrophic prokaryotic biomass production. Mean picocyanobacterial biomass during
430 the incubations (0.86 ± 0.18 μg C L^{-1}) was lower than heterotrophic prokaryotic
431 biomass (6.8 ± 1.1 μg C L^{-1}). Even if picocyanobacteria only represented from 2-20% of
432 total prokaryotic abundance, their contribution to leucine incorporation could be
433 considerable, as *Prochlorococcus* may display higher cell-specific incorporation rates
434 than heterotrophic bacteria because of their larger volume (40). The few existing
435 estimates indicate that *Prochlorococcus* may contribute up to 24-63% to the total
436 microbial plankton leucine incorporation into proteins (39-40).

437 We also found a significantly negative relationship between eCFs-LF and the
438 percentage of HNA prokaryotes (Table 2, Fig. 5B). As HNA prokaryotes appear to have
439 higher cell-specific leucine incorporation rates than LNA prokaryotes (42, 40) and tend
440 to be more susceptible to viral infection than LNA prokaryotes (43-45), the low eCFs-
441 LF could also be related to a high cell-specific leucine incorporation rates along with a
442 low net biomass increase of this bacterial functional group during the incubations. As
443 grazing was minimized by prefiltration we hypothesize that other factors, such as viral
444 lysis or apoptosis, could be responsible for low net biomass accumulation during our
445 incubations. The fact that picocyanobacterial abundance and the relative abundance of
446 HNA cells explained 80% of the variability observed in eCFs-LF in these oligotrophic

447 waters, suggest that prokaryotic community composition, and particularly these two
448 prokaryotic groups, have a strong influence on eCF estimates.

449 In conclusion, we have shown that whereas light exposure does not have a clear
450 effect on the leucine-to-carbon eCFs, the dilution pre-treatment tend to reduce the
451 carbon-to-leucine yield and promotes important changes in bacterial community
452 composition (assessed with ARISA fingerprinting) compared to unmanipulated
453 seawater samples. Filtration alone, on the other hand, allowed bacterial biomass
454 increase and did not imply important changes in bacterial community composition, thus
455 appearing to be an adequate experimental approach for deriving empirical conversion
456 factors, as the environmental characteristics would be closer to the *in situ* conditions
457 compared to the filtered and diluted treatments. We also provide a new perspective to
458 explain low eCFs in oceanic oligotrophic waters, in addition to the previously proposed
459 hypotheses of high protein turnover and leucine catabolism. We hypothesize that eCFs
460 variability patterns could be driven, in part, by low net biomass accumulation of highly
461 active prokaryotes during the incubations. As prokaryote biomass loss cannot be totally
462 avoided during the conversion factor experiments, the prokaryotic production rates
463 derived from the application of eCFs may not render gross biomass production rates,
464 which should be taken into account when constructing microbial carbon budgets.

465

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619

Table 1. Environmental conditions at the beginning of the leucine-to-carbon conversion factor experiments. Temperature (Temp), chlorophyll-*a* (Chla), phosphate concentration (PO_4^-), nitrate concentration (NO_3^-), and virus abundance correspond to unmanipulated seawater. Leucine incorporation rates, heterotrophic prokaryotic biomass (HPB), percentage of high nucleic acid content prokaryotes (%HNA), picocyanobacterial abundance and prokaryotic cell carbon content (C content) correspond to 0.8 µm-filtered seawater. Eq, equatorial, NA, not available.

EX-Ocean	**Temp (ºC)**	**Chla (µg L^{-1})**	**PO_4^- (µmol l^{-1})**	**NO_3^- (µmol l^{-1})**	**Leucine incorporation (pmol leu L^{-1} h^{-1})**	**HPB (µg C l^{-1})**	**%HNA**	**Picocyanobacterial abundance (x 10^4 cell ml^{-1})**	**C content (fg C $cell^{-1}$)**	**Virus abundance (10^6 ml^{-1})**
1-N Atlantic	24.80	0.18	NA	0.509*	30.2	6.39	41	9.68	13.2	13.71
2-Eq Atlantic	27.50	0.15	0.078	NA	168.6	11.14	51	13.30	13.6	5.92
3-S Atlantic	22.50	0.03	0.170	0.361*	34.5	3.84	25	1.37	13.1	NA
4-S Indian	25.90	0.09	0.033	0.262*	22.4	4	47	3.31	12.3	1.54
5-S Indian	21.70	0.07	0.019	0.191	6.3	2	38	5.74	9.8	2.99
6-S Pacific	24.00	0.13	0.089	0.143	5.5	4.12	53	6.23	12.9	3.10
7-Eq Pacific	28.30	0.18	0.319	2.280	2362.4	14	51	6.87	15.9	9.91
8-N Pacific	24.02	0.09	0.083	0.028	6.2	7.67	37	1.51	12.8	4.35
9-N Pacific	28.20	0.21	0.229	0.377	68.3	7.21	38	15.00	10.2	43.44
10-N Atlantic	28.70	0.09	0.068	0.340	35.8	6	27	4.67	12.7	12.50

*Data from 7-17 m depth within the mixed layer

Table 2. Pearson correlation coefficients between eCFs in light + filtration + dilution (LFD), dark + filtration +dilution (DFD) and light + filtration (LF) treatments and different environmental factors. HPB, heterotrophic prokaryotic biomass, %HNA, percentage of high nucleic acid content prokaryotes, C content, prokaryotic cell carbon content. *, $p<0.05$; **, $p<0.01$; ns, not significant. N=10, except for Phosphate (N=9).

	eCFs-LFD	eCFs-DFD	eCFS-LF
Temperature	ns	ns	ns
Chlorophyll-*a*	ns	ns	-0.67*
Phosphate	ns	ns	ns
Ln Nitrate	ns	ns	ns
Leucine incorporation	ns	ns	ns
HPB	ns	ns	ns
% HNA	ns	ns	-0.75*
Log picocyanobacterial abundance	ns	ns	-0.80**
C content	ns	ns	ns
Virus abundance	ns	ns	ns

Figure legends

Figure 1. Map of sampling locations where leucine-to carbon empirical conversion factor experiments were conducted.

Figure 2. Dendrogram based on Automated Ribosomal Intergenic Spacer Analysis (ARISA) profile similarities of unmanipulated seawater at the beginning of the experiments. Dashed lines represent statistically significant clusters ($p < 0.05$) according to the Simprof test. Eq, equatorial, Atl, Atlantic, Ind, Indian, Pac, Pacific.

Figure 3. Heterotrophic prokaryotic biomass (HPB) versus accumulated leucine incorporation for the light + filtration (LF), light + filtration + dilution (LFD) and dark + filtration + dilution (DFD) treatments in each of the 10 experiments. Eq, equatorial, Atl, Atlantic, Ind, Indian, Pac, Pacific. Error bars represent the standard error from duplicates.

Figure 4. Mean leucine-to-carbon empirical conversion factors (eCFs) obtained for the light + filtration (eCFs-LF), light + filtration + dilution (eCFs-LFD) and dark + filtration + dilution (eCFS-DFD) treatments in each of the 10 experiments. Eq, equatorial, Atl, Atlantic, Ind, Indian, Pac, Pacific. Error bars represent the standard error from duplicates.

Figure 5. Relationship between eCFs-LF and (A) picocyanobacterial abundance or (B) the percentage of HNA prokaryotes. Eq, equatorial, Atl, Atlantic, Ind, Indian, Pac, Pacific.

Figure 6. Dendrogram based on Automated Ribosomal Intergenic Spacer Analysis (ARISA) profile similarities of seawater samples from light + unmanipulated (LC), light + filtration (LF), light +filtration + dilution (LFD) and dark + filtration +dilution (DFD) treatments at the end of the experiments. Dashed lines represent statistically significant

clusters ($p < 0.05$) according to the Simprof test. Eq, equatorial, Atl, Atlantic, Ind, Indian, Pac, Pacific.

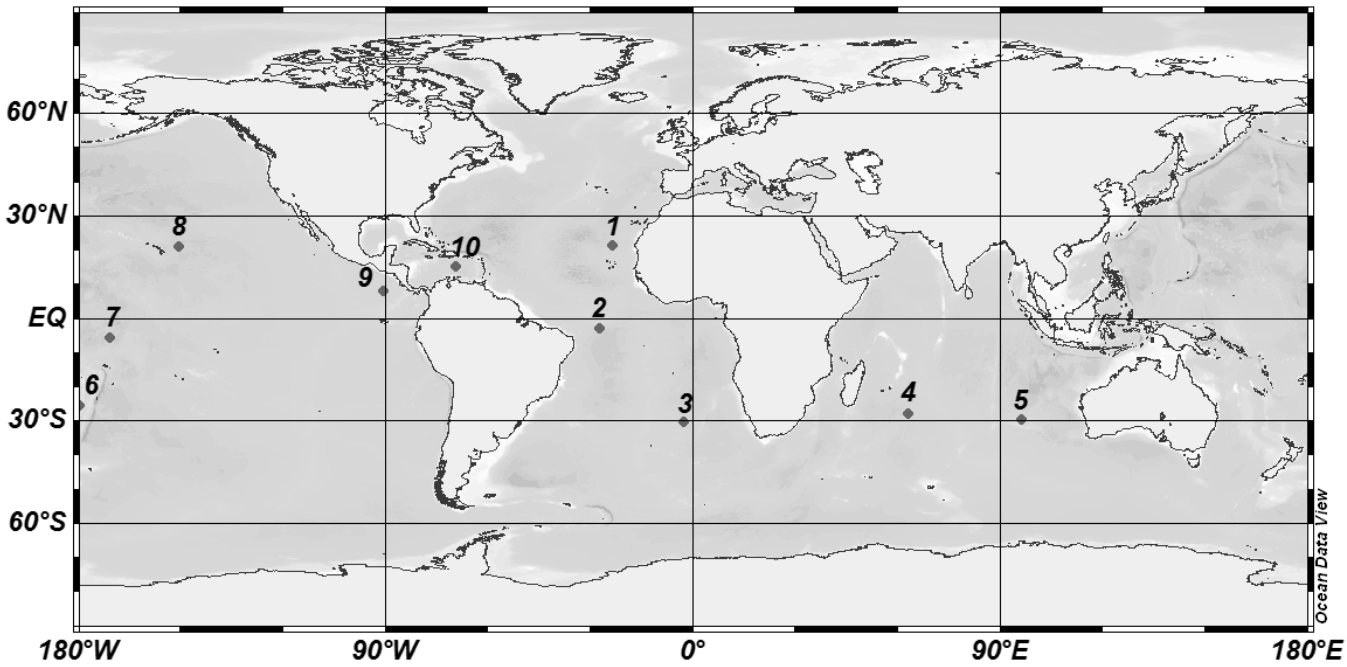

Figure 1

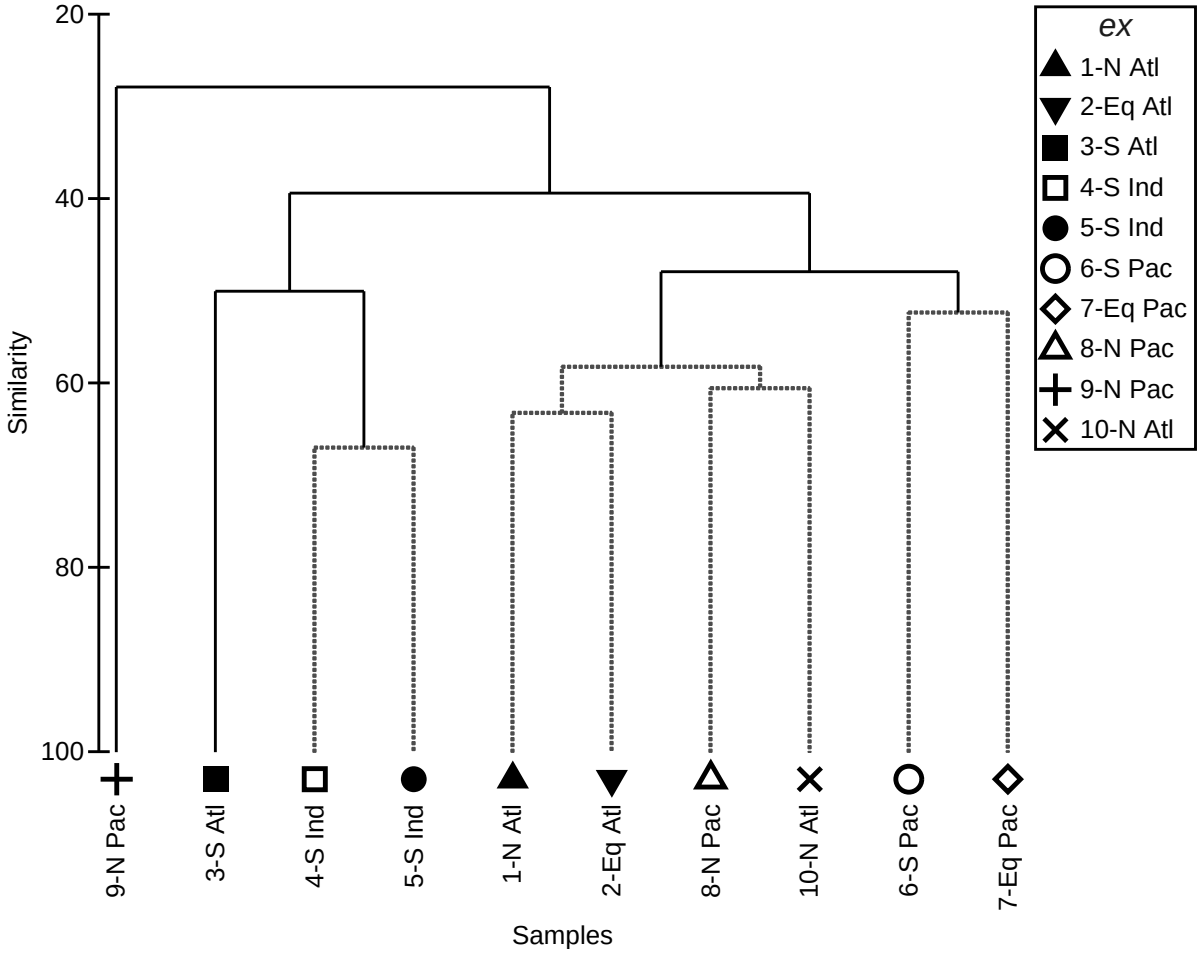
20
40
60
80
100
Similarity
9-N Pac
3-S Atl
4-S Ind
5-S Ind
1-N Atl
2-Eq Atl
8-N Pac
10-N Atl
6-S Pac
7-Eq Pac
Samples
ex
1-N Atl
2-Eq Atl
3-S Atl
4-S Ind
5-S Ind
6-S Pac
7-Eq Pac
8-N Pac
9-N Pac
10-N Atl

Figure 2

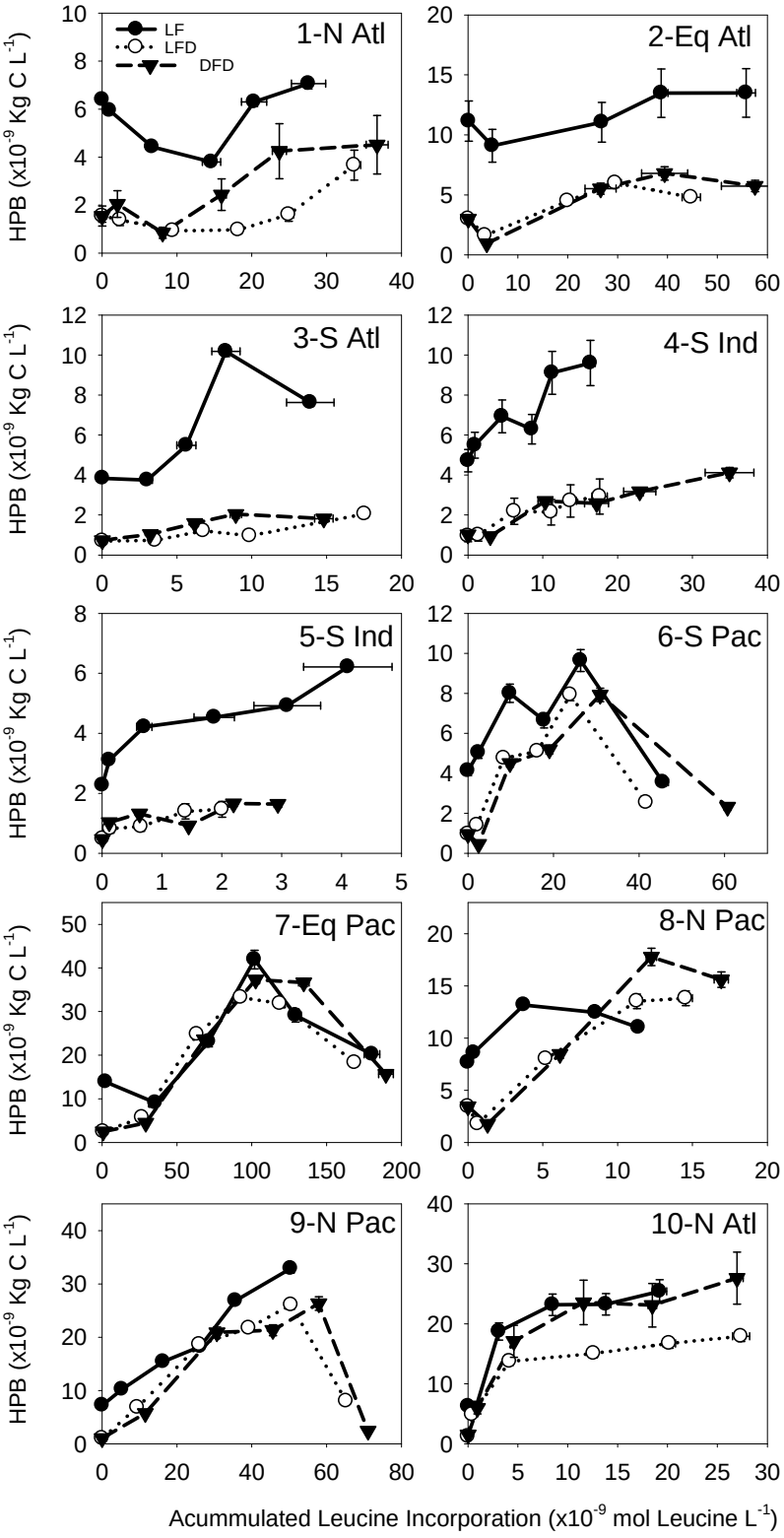
LF
LFD
DFD
1-N Atl
2-Eq Atl
3-S Atl
4-S Ind
5-S Ind
6-S Pac
7-Eq Pac
8-N Pac
9-N Pac
10-N Atl
HPB (x10-9 Kg C L-1)
Acummulated Leucine Incorporation (x10-9 mol Leucine L-1)

Figure 3

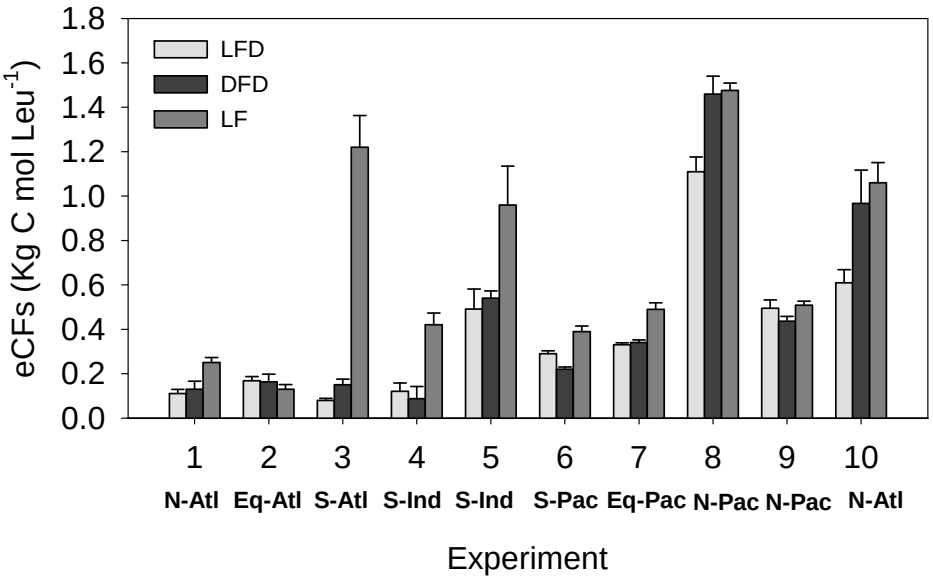
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
eCFs (Kg C mol Leu-1)
LFD
DFD
LF
1
2
3
4
5
6
7
8
9
10
N-Atl
Eq-Atl
S-Atl
S-Ind
S-Ind
S-Pac
Eq-Pac
N-Pac
N-Pac
N-Atl
Experiment

Figure 4

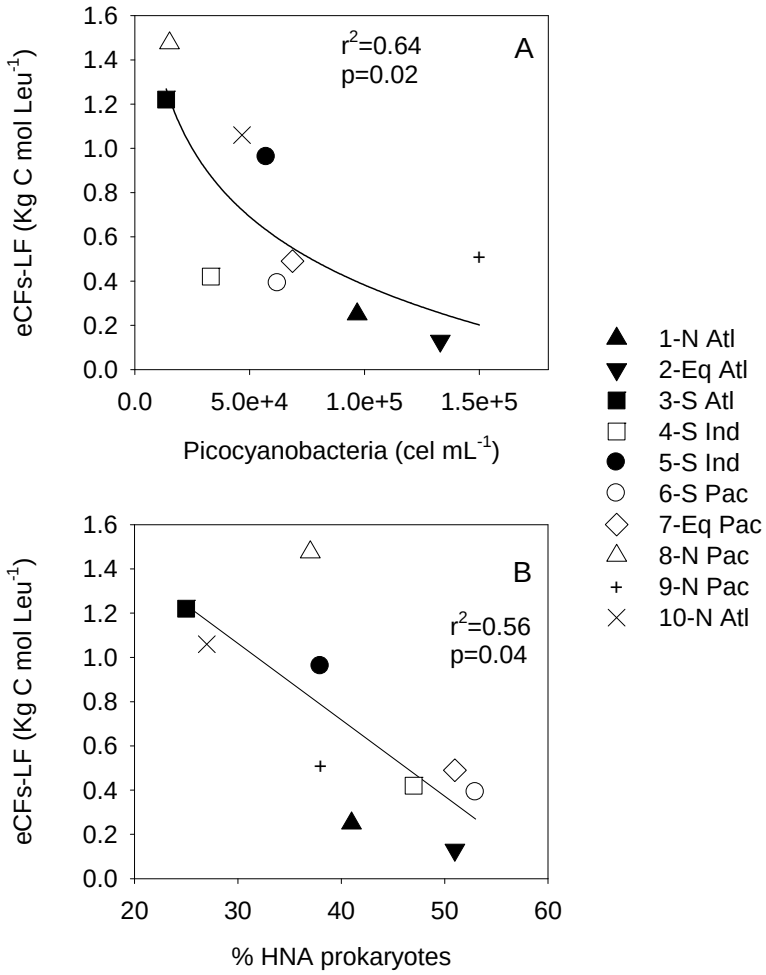
A
r2=0.64
p=0.02
eCFs-LF (Kg C mol Leu-1)
Picocyanobacteria (cel mL-1)
B
r2=0.56
p=0.04
% HNA prokaryotes
1-N Atl
2-Eq Atl
3-S Atl
4-S Ind
5-S Ind
6-S Pac
7-Eq Pac
8-N Pac
9-N Pac
10-N Atl

Figure 5

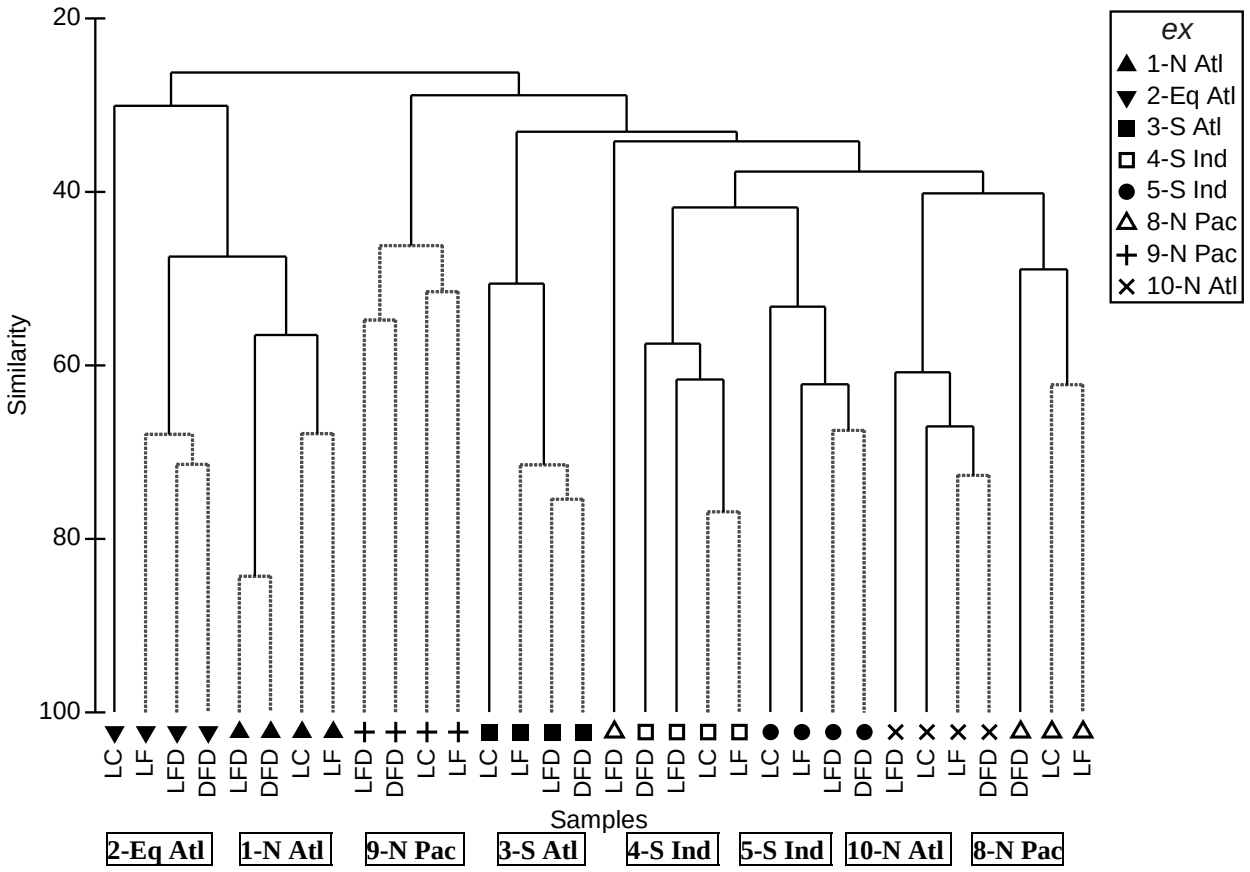
Similarity
20
40
60
80
100
ex
1-N Atl
2-Eq Atl
3-S Atl
4-S Ind
5-S Ind
8-N Pac
9-N Pac
10-N Atl
LC LF LFD DFD LFD DFD LC LF LFD DFD LC LF LC LF LFD DFD LFD DFD LFD LC LF LC LF LFD DFD LFD LC LF DFD DFD LC LF
Samples
2-Eq Atl
1-N Atl
9-N Pac
3-S Atl
4-S Ind
5-S Ind
10-N Atl
8-N Pac

Figure 6